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14. ABSTRACT: Of the genetic alterations associated with breast cancer, changes in p53 are the most frequently identified and a subset of these changes destabilizes the p53 core domain structure. The overall goal of our studies is to identify small molecule compounds that bind and stabilize this subset of tumor-derived p53 mutants. We anticipate that the identification of such compounds will serve as a scaffold for the preparation of small molecule drugs for the treatment of p53-mediated breast cancer. Towards this goal, we have employed a Multiple Solvent Crystal Structures (MSCS) technique to identify a p53 binding sites for the small molecule compound tris (hydroxymethyl)aminomethane (Tris) and have used both solution studies and molecular dynamics simulations to show that Tris binding increases the stability of the p53 core domain. We have also carried out virtual screening (in silico) to identify Tris analogues that are predicted to have improved p53 core domain binding and stability properties. In the coming year, we will continue the virtual screening studies and test our virtual screening "hits" in solution for improved p53 core domain binding and stability. We will also cocrytallize the p53 core domain with the Tris analogues that show the most favorable properties for second generation structure-based optimization of these compounds.

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(5) INTRODUCTION

Of the genetic alterations associated with breast cancer, changes in p53 are the most frequent and identified in 20-40% of all cases (Borresen-Dale, 2003; Ziyaie et al., 2000). In fact, approximately half of the major forms of cancer contain p53 mutations, and the vast majority of these cluster in conserved regions or "hot spots" (Hainaut and Hollstein, 2000). Missense mutations leading to amino acid changes are the most common p53 alterations in breast cancer, as in other tumors (Hainaut and Hollstein, 2000). Together, these observations suggest a requirement for a putative oncogenic contribution conferred by many TP53 mutations in breast cancer, and imply that the development of small molecule compounds that may bind and reactivate the protein product of tumor-derived TP53 mutations may have therapeutic use for the treatment of breast cancer.

The TP53 gene encodes the p53 protein that regulates the transcription of a number of genes involved in cell-cycle arrest and induction of apoptosis in response to cellular or genotoxic stress such as DNA damage or hypoxia (Bargonetti and Manfredi, 2002). The transcriptional activity of p53 is mediated by a tetrameric form of the protein that binds DNA in a sequence-specific fashion to activate or repress the transcription of target genes (El-Deiry et al., 1993; Friedman et al., 1993; Halazonetis and Kandil, 1993; Stenger et al., 1994). p53 contains four functionally distinct domains: a N-terminal transcriptional activation domain (residues 1 to 44), a central core (residues 102 to 292) containing a DNA binding domain, a tetramerization region (residues 320 to 356), and a regulatory domain (residues 356-393) (Cho et al., 1994; Pavletich et al., 1993; Wang et al., 1993). The vast majority of tumor-derived p53 mutations are localized to the p53 core domain (Cho et al., 1994). The X-ray crystal structure of the monomeric core domain of p53 bound to DNA has provided invaluable insights into how several tumor-derived mutations in p53 disrupt its activity (Cho et al., 1994). Specifically, these studies reveal that the tumor-derived p53 mutations that are localized to the core domain result in two different classes of p53 protein alterations: (1) reduced protein thermostability mutations and (2) mutations that directly disrupt protein-DNA contacts. Both classes of mutations functionally compromise the ability of p53 to carry out its normal tumor suppression function and thus contribute to neoplasia. The goal of our studies is to identify lead compounds that bind and stabilize the subset of tumor-derived stability mutants within the p53 core domain. We anticipate that the identification of such compounds will serve as a scaffold for the preparation of small molecule drugs for the treatment of p53-mediated breast cancer.

The Specific Aims of the proposal are to (1) Determine the high resolution X-ray crystal structure of the p53-core domain bound to a stabilizing peptide called FL-CDB3, (2)Use the Multiple Solvent Crystal Structures (MSCS) technique, to identify novel p53 stabilization sites, (3) Use the structural information of aims 1 and 2 as a scaffold for using computational strategies for the further development of small molecule compounds and peptides for the reactivation of tumor derived p53 mutants, and (4) Functionally characterize the p53-stabilizing and p53-reactivation properties of the molecules derived from aim 3, and determine their structures in complex with p53.

(6) BODY

During the first year of the funding period we completed Aim1 (Tasks 1-2), Aim2 (Tasks 3-4) and Task 7 of Aim 4. For Aim1, we determined the 2.5Å resolution structure of crystals that were prepared by mixing the p53 core domain with the Fl-CDB3 peptide. Unfortunately, the structure did not reveal ordered electron density for the peptide. Subsequent experiments involved soaking preformed p53 core domain crystals with peptide which also produced a structure in which no ordered density for the peptide could be identified. We conclude that the FL-CDB3 peptide does not bind p53 in a unique location and conformation and therefore that it is not possible to characterize a structure of a p53/FL-CDB3 complex. This is consistent with recent observations that have been made by Fersht and coworkers (Friedler et al., 2005).

For Aim 2, we determined the structure of the p53 core domain bound to two small molecule compounds, isopropanol and tris(hydroxymethyl)aminomethane (Tris), bound to the L1 loop and to a region of p53 shown to be important for repair of a subset of tumor-derived p53 mutations, respectively (Figure 1). Correlating with the significance of the p53-Tris interactions seen in the crystals, we carried out equilibrium denaturation experiments that demonstrate that Tris increases the thermodynamic stability of the mouse p53 core domain by about 0.74 kcal/mol (Figure 2), suggesting that the p53/Tris complex may provide a useful scaffold for the structure-based design of p53 stabilizing compounds. The details of our findings during the first year of funding are described in our last report.

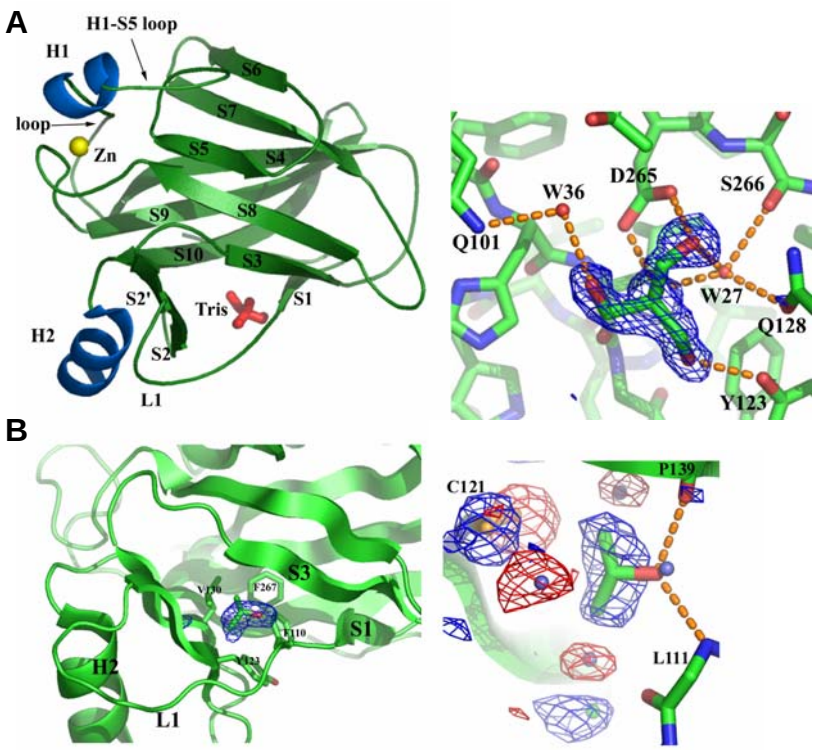

Figure 1. A) **Structure of the p53 core domain/Tris complex.** Left- Overall structure of the complex, Right – close-up of the complex highlighting omit-density (blue chicken wire) and protein-mediated hydrogen bonds (orange dotted lines) mediated by the Tris molecule. B) **Structure of the p53 core domain/isopropanol complex.** Left – Overall structure of the complex. Right – close-up of the complex using the same color-coding as in A. The red chicken wire represents displaced water molecules upon isoproponal binding.

During the second year of the funding period we have completed Aim3 (Tasks 5-6). For this Aim, we have quantitatively analyzed the stabilizing effect of Tris binding to p53 using computational (in silico) techniques. We have

also used computational strategies to identify Tris-like molecules that are predicted to bind p53 with higher affinity and potentially to increase the degree of small molecule stabilization.

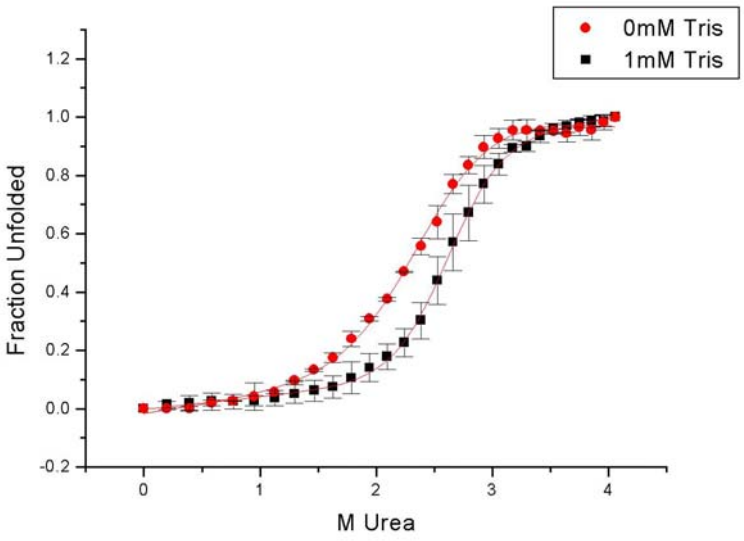

Figure 2. Urea induced unfolding of the p53 core domain in the absence (red) or presence of 1 mM Tris. Fraction unfolded is monitored by an increase of tryptophan florescence from a tryptophan that is buried in the folded protein.

The behavior of the p53 core domain both alone and in complex with Tris was studied by molecular dynamics simulation to account for protein flexibility and conformational changes in solution environment. Briefly, the structures were subjected to 5.0 ns MD simulations using the program GROMACS 3.3 (Van Der Spoel et al., 2005). The RMSD values of backbone atoms from their initial positions (t = 0 ps) were used to measure protein stability and to gain

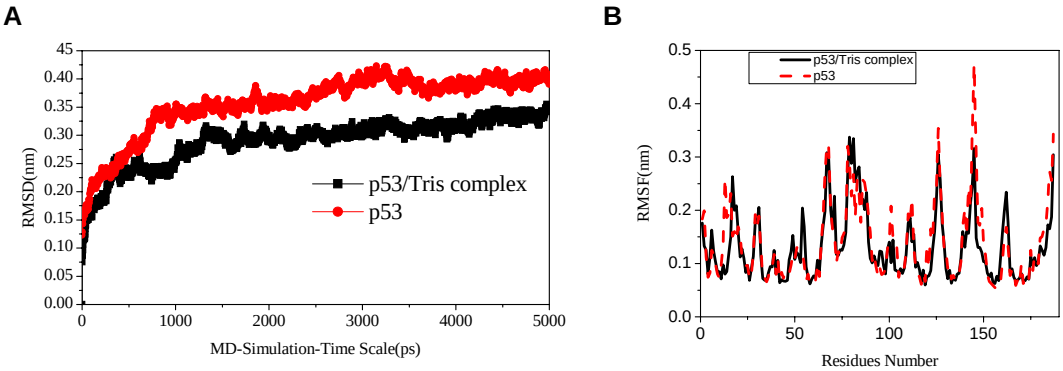

Figure 3. Molecular dynamics simulations of the p53 core domain in the presence and and absence of bound Tris. (A) Average RMSD over the picosecond time scale. (B) The RMSD from A is plotted as a function of residue number. Results for the p53/Tris complex and p53 alone are plotted in red and black, respectively.

insight into possible structural fluctuation. The time evolution of the backbone atom RMSD values for both systems are presented in Figure 3A. In this plot, a sharp rise is observed during the first 200 ps in RMSD of all residues and then it flattens out. The magnitude of these RMSD curves, however, does not continue to increase after about 1.5 ns of MD simulation, implying that both systems are stable over this timescale. The

average RMSD values are about 3.0 and 3.7 Å, for the p53/Tris complexes and p53, respectively. This is indicative of the relative stability of the p53 core domain containing the bound Tris molecule. In addition, this trend is also apparent in the analysis of residue-wise RMS fluctuations, shown in Figure 3B which shows that the stabilizing effect of Tris on the p53 core domain is distributed throughout the p53 core domain. It has been proposed that an increase in protein stabilization is correlated with a decrease in the conformational flexibility of the protein (Matthews et al., 1987). Therefore, is likely that Tris binding stabilizes the p53 core domain by decreasing its conformational flexibility which is in agreement with the equilibrium denaturation results presented in Figure 2.

Using Tris as a lead compound for p53 core domain stabilization, we carried out a virtual screening using the SPECS (http://www.specs.net/) and TimTec (http://blaster.docking.org/zinc/) databases. Screening was performed on the Pittsburg Supercomputing Center (www.psc.edu) using a Linux server in our lab. Since a scoring function has not yet been developed to reliably and consistently rank and quantitate ligand-protein energies, a heuristic docking and consensus scoring strategy was used in the virtual screening. In this particular case, the program DOCK4.0 (Morris et al., 1998) was employed for the primary screening with a radius of 6 Å around the Tris molecule. During the molecular docking calculations, Kollman-all-atom charges were assigned to the protein, and Geisterger-Hückel charges were assigned to tris molecules due to lack of proper Kollman charges. The conformational flexibility of the compounds from the databases were considered in the docking procedure and the DOCK suite was used to evaluate the results using a shape scoring function and/or a function approximating the ligand-receptor binding energy. Following the initial orientation and scoring evaluation, a grid-based rigid body minimization was carried out for the ligands to locate the nearest local energy minimum within the receptor binding site. The position and conformation of each docked molecule was optimized using single anchor search and a torsion minimization method in DOCK4.0. Fifty configurations per ligand building a cycle and 50 maximum anchor orientations were used in the anchor-first docking algorithm. All docked configurations were energy minimized using 100 maximum iterations and 1 minimization cycle.

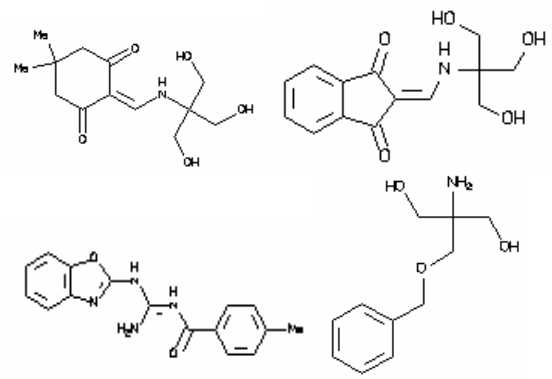

Figure 4. Structure of 4 Tris like compounds that showed favorable p53 docking properties.

Following molecule selection based on the docking results, the top 40000 molecules from each database were selected for further analyses. These molecules were re-scored using the program SLIDE, XSCORE and the scoring function of AutoDock3.0. Based on the

second scoring results, 13 compounds (10 from the SPECS database and 3 from the TimTec database) were selected for further analysis using solution studies. Some representative compounds from this set are shown in Figure 4.

(7) KEY RESEARCH ACCOMPLISHMENTS

- We have demonstrated, both in solution and in silico, that Tris binding stabilizes the core domain of p53 and therefore Tris qualifies as a suitable lead compound for the structure-based optimization of p53 stabilizing compounds with possible therapeutic application for p53-mediate breast cancer.
- We have identified second generation Tris-like p53 stabilizing compounds in silico that are suitable for further investigation of their p53 stabilization properties in solution.

(8) REPORTABLE OUTCOMES

A manuscript describing these studies is in preparation.

(9) CONCLUSIONS

In the coming year we will carry out Aim 4 (Tasks 7-9) to further characterize the Tris analogues that we identified through our virtual screening procedure, in solution for p53 binding and stability properties. Compounds that show increased solution binding and stability relative to Tris will be cocrystallized with the p53 core domain. We will also continue our virtual screening to identify additional Tris analogues (hits) that are predicted to bind and stabilize the p53 core domain and we will further filter these hits for compounds that show good solubility characteristics. Additional "hits" will be further analyzed in solution as described above.

The structure-based drug design approach (often called "rational drug design"), that we are using towards the development of small molecule compounds that might restore function to tumor-derived p53 mutants, is a recently exploited and particularly powerful strategy which uses protein structural information to specifically design small peptides or non-peptidic molecules that modulate the activity of a protein of interest (Garrett and Workman, 1999; Huang, 2000; Jackson, 1997; Oakley and Wilce, 2000; Tada et al., 1999; Wang et al., 1999; Wieczorek et al., 1996). This strategy has shown considerable promise, already yielding clinically useful peptides and compounds (Amzel, 1998; Gane and Dean, 2000; Kirkpatrick et al., 1999; Klebe, 1998; Kubinyi, 1998; Lunney, 1998; Roe et al., 1998; Sehgal, 2002) as well as several other compounds currently in clinical trials (Klebe, 1998). Based on our encouraging results to date, we propose that a structure-based approach is an effective strategy of achieving our ultimate goal of developing p53-targeting drugs that will have clinical application for the treatment of p53-mediated breast cancer.

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